Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 5325–5329, July 1988
Neurobiology

Clone-forming ability and differentiation potential of migratory neural crest cells

(avian embryo/cell lineage/tyrosine hydroxylase/neuropeptides/3T3 cell feeder layers)

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Communicated by Howard Green, April 4, 1988

ABSTRACT **The neural crest of vertebrate embryos gives rise to a variety of differentiated cell types, including neuronal and non-neuronal cells of peripheral ganglia (sensory and autonomic), pigment cells, and mesectodermal derivatives. Neural crest cells were taken from quail embryos at the level encompassing mesencephalon and metencephalon and the developmental potentials were evaluated by culturing them as single cells on 3T3 feeder layers. Such conditions proved to be particularly favorable for survival, proliferation, and differentiation of quail neural crest cells. Two hundred and forty-three clones that contained from 1 to >20,000 cells were analyzed after 7–10 days of culture. Phenotype analysis provided evidence for the existence of cells with extremely diverse developmental potencies. A few committed neuron progenitors were observed as well as some pluripotent cells, able to differentiate into several types of neurons, non-neuronal cells, and melanocytes, and many cells with intermediate developmental potencies. These cloning experiments revealed the striking heterogeneity of migrating neural crest cells in terms of their capacity for differentiation and their potential for proliferation.**

The neural crest (NC) is a transitory and pluripotent structure of vertebrate embryos. It forms from the lateral ridges of the neural plate as the ridges join during the closure of the neural tube. Shortly afterward the cells of the NC migrate extensively throughout the embryo and give rise to a number of differentiated cell types that include all the neurons of the peripheral ganglia (i.e., sensory, sympathetic, parasympathetic, and enteric) except those of the distal ganglia of cranial nerves V, VII, IX, and X, which originate from ectodermal placodes. The satellite glial cells associated with these neurons and the Schwann cells of peripheral nerves are also of NC origin. Moreover, the pigment cells of the body, apart from those of the pigmented retina, arise from the NC, as do certain endocrine and paraendocrine cells (adrenomedullary cells, calcitonin-producing cells, and carotid body type I cells). In addition, the NC yields mesectodermal derivatives that form connective and skeletal structures of the face and whose precursor cells are restricted to the cephalic region of the neural axis in higher vertebrates (1).

NC development thus involves the segregation of various cell lineages from the initial population of migratory cells. It has been shown that the microenvironment in which the cells differentiate has an important influence on the phenotypes that they express (2). Various investigations from our laboratory (3) and other laboratories (4–7) have led to the hypothesis that environmental factors do not act on a homogeneous population of pluripotent NC cells but rather on cells that are heterogeneous with respect to their developmental potentials. The most unambiguous approach to investigate the differentiation capacity of a given embryonic cell is to identify the phenotypes of its progeny. One way to achieve this in higher vertebrates is to label a progenitor cell with a stable transmittable marker *in vivo*—for example, with an inheritable retroviral marker, as was done in newborn rat retina (8). Alternatively, a single isolated precursor cell could be cultured under conditions permitting full expression of its proliferation and differentiation potentials. Clonal analysis of vertebrate NC cell development showed (5, 9–12) that the progeny of individual truncal NC cells varied qualitatively and quantitatively, indicating differences in commitment at the time the cells were cultured. In these experiments, only three phenotypes were considered—namely melanocytes, adrenergic cells, and "other" cells (expressing neither set of traits). To extend the analysis of the developmental potentialities of single NC cells *in vitro*, we used the system described by Green and coworkers (13, 14) that was devised to culture and clone human keratinocytes. Cranial NC cells were seeded individually on feeder layers of growth-inhibited 3T3 cells in a complex culture medium. Under these conditions, NC cells produced a wide variety of clones, in terms of their size and phenotypic properties, thus revealing the remarkable heterogeneity of these cells, many of which are pluripotent.

MATERIALS AND METHODS

The NC was removed, over a length corresponding to mesencephalon and metencephalon from 9- to 13-somite quail embryos as described (15) and dissociated into single cells in a solution of 0.025% trypsin (Difco) in Ca^{2+}/Mg^{2+}-free isotonic phosphate-buffered saline for 5 min at 37°C. After washing in culture medium, the cells were either plated as mass cultures (in which case all the cells resulting from the dissociation of an entire NC were cultured) or as single cells. Individual cells were identified by using an Olympus CK2 inverted microscope (10× phase-contrast objective). Randomly selected cells were aspirated with an elongated Pasteur pipet from a dilute suspension of 500–1000 cells—corresponding to the dissociation of the two NCs of the same embryo. All cells were cultured on established layers of Swiss 3T3 cells (16) whose growth had been arrested by treatment for 2–3 hr with mitomycin (4 μg/ml) (Sigma). Mass cultures of NC cells were grown in 35-mm dishes (Nunc). Unless otherwise stated, single NC cells were seeded either in Lab-Tek chamber slides (Miles) or in 4-well plates (Nunc). In all cases, the culture medium consisted of Ham's F-12 nutrient mixture/Dulbecco's modified Eagle's medium/BGjb medium [6:3:1 (vol/vol); Irvine Scientific] supplemented with 10% (vol/vol) fetal calf serum (Biological Industries), 2% (vol/vol) embryo extract [from 11-day

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Abbreviations: NC, neural crest; mAb, monoclonal antibody; TyrOHase, tyrosine hydroxylase; SP, substance P; VIP, vasoactive intestinal polypeptide; NF, neurofilament.

chicken embryos (ref. 25; the same frozen batch was used for all experiments)], adenine (24.3 ng/ml), hydrocortisone (0.4 μg/ml), insulin (5 μg/ml), triiodothyronine (13 ng/ml), transferrin (10 μg/ml), epidermal growth factor (10 ng/ml), isoproterenol (0.25 μg/ml), and choleragen (8.4 ng/ml). All reagents were from Sigma. The cultures were maintained at 37°C in an atmosphere of 5% CO_2/95% air. The medium was changed every 2 or 3 days.

At specified times, the cultures were fixed in a solution of 4% (wt/vol) paraformaldehyde and Ca^{2+}/Mg^{2+}-free isotonic phosphate-buffered saline (pH 7.4). Clones were detected and counted after staining with Hoechst nuclear dye (1 μg/ml; catalog number 33342; Serva, Heidelberg), which allows NC cells to be distinguished from the 3T3 cells (Fig. 1). Phenotypes were identified by indirect immunocytochemistry with various antibodies: rat anti-substance P (SP) monoclonal antibody (mAb) (Seralab, Crawley–Down, England), mouse anti-tyrosine hydroxylase (TyrOHase) mAb (M. Fauquet and C. Ziller, personal communication), mouse anti-neurofilament (NF) protein mAb (Immunotech, Marseille, France), and rabbit anti-vasoactive intestinal polypeptide (VIP) serum (17). In addition, HNK1, a mouse mAb (18) recognizing a majority of migrating NC cells and later in development neuronal and non-neuronal cells of the peripheral nervous system (19) was used. HNK1 is not absolutely specific for NC-derived lineages since, at later developmental stages, the glycosylated epitope identified by this mAb is carried by other cell types (20). However, in the context of these cultures of cephalic NC cells, it constitutes a useful marker for most peripheral nervous system cellular components. The secondary antibodies used were fluorescein or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA), goat anti-rabbit immunoglobulin (Nordic, Tilburg, The Netherlands), or fluorescein isothiocyanate-conjugated rabbit anti-rat IgG (Zymed Laboratories, Burlingame, CA). Clones were analyzed as follows: Clones were incubated with anti-SP and then with anti-TyrOHase. Clones positive for SP and negative for TyrOHase were incubated with anti-NF. Clones negative for SP and TyrOHase were incubated with anti-NF and then with anti-VIP. Finally, all clones were treated with HNK1. Melanocytes were identified by the presence of melanin visible by phase-contrast microscopy. Cultures in which procartilage formations had been tentatively identified by phase-contrast microscopy were stained with an acidic solution of toluidine blue (21).

RESULTS

Mass Cultures. Several mass cultures of dissociated NC cells were analyzed after 3–18 days *in vitro* on 3T3 cell feeder layers. Proliferation of NC cells was dramatic. From the beginning of the culture, NF immunoreactivity could be detected in multipolar neurons, many of which showed SP-like immunoreactivity; later (starting at 6 days), VIP-like immunoreactivity was associated with some NF^+ cells. After 4 days, TyrOHase-containing cells appeared, most of them being NF^-, but interestingly 2–10% of $TyrOHase^+$ cells were also SP^+ or VIP^+. In all cultures, regardless of the time at which they were observed, $HNK1^+$ cells with a neuronal or non-neuronal morphology were numerous. After 3 days, melanocytes appeared in the cultures; and after 6 days, formations of procartilage, showing metachromaticism with toluidine blue, could be seen.

These results showed that this culture system provides NC cells with conditions favorable for proliferation and differentiation. Consequently, cloning of NC cells was envisaged.

Clonal Cultures. Clonal culture conditions (wells >100 mm^2 for a 10-day culture period) were such that they favored detection of large clones. From eight experiments in which 736 single cells were seeded, a total of 190 clones were detected after 10 days. Seventeen clones displaying none of the chosen markers were eliminated because they may have been derived from non-NC cells. The 173 clones retained correspond to a mean cloning efficiency of 24% (range, 10–41%).

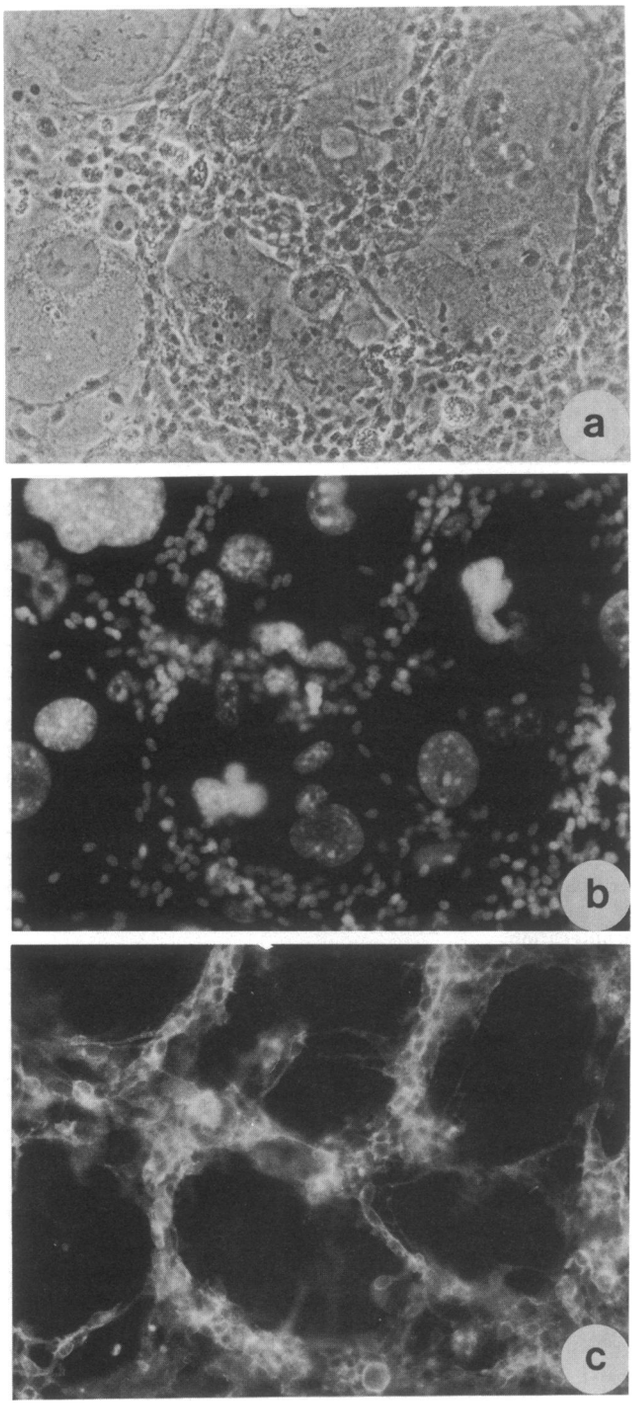

FIG. 1. Phase-contrast micrograph (*a*), micrograph of Hoechst stained colony (*b*), and micrograph of HNK1 stained colony (*c*). Micrographs show ≈1/30 of a typical 10-day clone containing ≈10,000 cells, some of which contained SP or TyrOHase. The Hoechst stain allowed us to distinguish NC cells (small oval nuclei) from 3T3 cells (large and irregular spotted nuclei). NC cells are stained by HNK1. (×260.)

These 173 clones contained from 1 cell to >20,000 cells (Figs. 1 and 2). More than 80% contained >100 cells; the largest clones covered as much as 12 mm^2. The NC colonies, usually round in shape, formed a network of predominantly spindle-shaped cells organized around 3T3 cells (Fig. 1). Cell

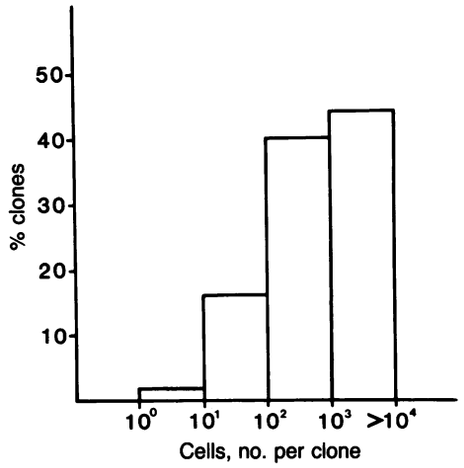

FIG. 2. Distribution of 10-day clonal cultures grown in large wells (n = 173) according to their cell number.

density was variable and could reach 2×10^3 cells per mm^2. In a given clone, however, cell density was quite uniform.

We first identified clones with neuronal cells by the presence of neurotransmitter-related molecules (TyrOHase) or neuropeptides (SP and VIP) (Fig. 3). Anti-NF staining did not reveal any other neuronal cells. Such phenotypes were identified in 76 clones (44% of the total clones), all of which contained >100 cells and 70% of which contained >1000 cells. We classified these clones into seven types by the presence of cells immunoreactive for SP, TyrOHase, or VIP (Fig. 4), or the presence of melanocytes. Neurons were always accompanied by non-neuronal cells that stained with HNK1. Sixty-one clones contained at least two neuronal markers (SP and TyrOHase or TyrOHase and VIP). We were not able to ascertain whether VIP$^+$ cells were also present in cultures that contained SP$^+$ cells for technical reasons resulting from the specificities of the corresponding secondary antibodies. The most frequent type of neuron-containing clone included cells expressing SP and TyrOHase separately, in distinct subpopulations, or colocalized to the same cells (Fig. 4). Melanocytes were observed in 6 neuronal clones and were present in clones with and without TyrOHase$^+$ cells. Products related to mesectodermal derivatives of the cephalic NC, such as procartilage, were never detected in clones containing neurons.

In the remaining 97 clones (56% of the total) we could not identify any neuron-related phenotype. Two of them contained melanocytes accompanied by a mixture of HNK1$^+$

TYPE OF CLONE	PHENOTYPES SP	TH	VIP	HNK1	MEL	CART	No CELL TYPES	No CLONES	
N + nN	●	●	?	●	●	○	⩾ 4	3	44%
	●	●	?	●	○	○	⩾ 3	56	
	○	●	●	●	○	○	⩾ 3	2	
	○	●	○	●	●	○	⩾ 3	2	
	○	●	○	●	○	○	⩾ 2	9	
	●	○	?	●	●	○	⩾ 3	1	
	●	○	?	●	○	○	⩾ 2	3	
nN	○	○	○	◐	●	○	⩾ 3	2	56%
	○	○	○	◐	○	●	⩾ 3	7	
	○	○	○	◐	○	○	⩾ 2	29	
	○	○	○	●	○	○	⩾ 1	59	

FIG. 3. Classification of the clones by cells expressing various phenotypic markers. N, neuron; nN, non-neuronal cell; MEL, melanocyte; CART, procartilage; TH, TyrOHase; No, number. ●, marker present in some (or all in the case of HNK1) cells; ○, marker absent from all cells; ?, not determined; ◐, mixture of HNK1$^+$ and HNK1$^-$ cells.

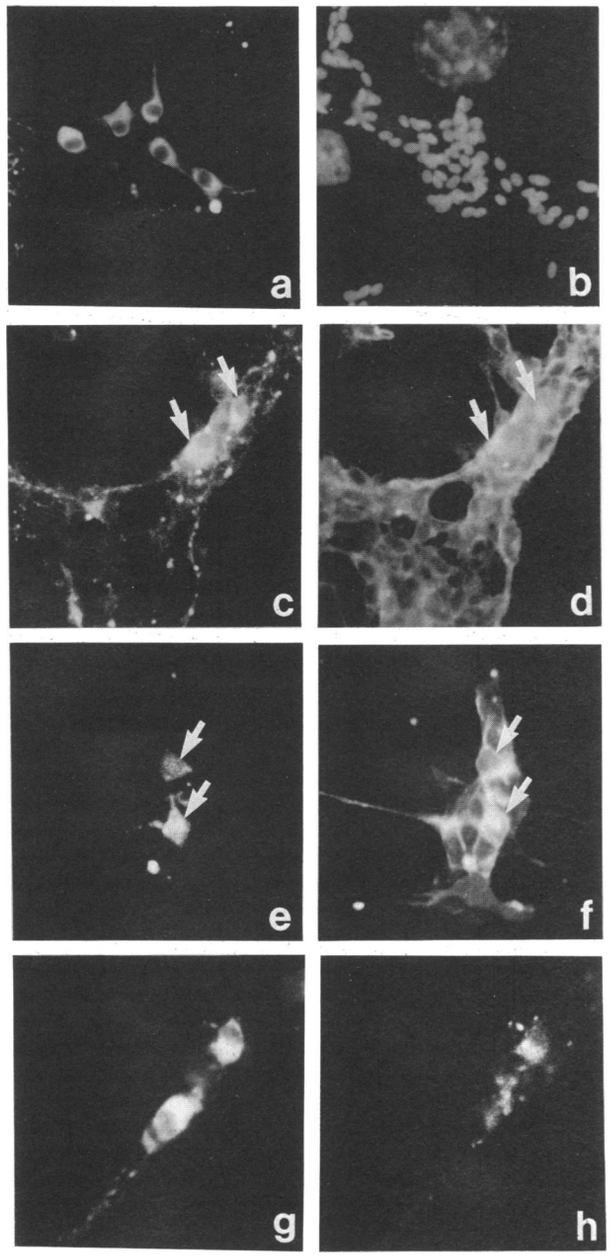

FIG. 4. Neuronal and non-neuronal phenotypes present in 10-day clones. Each horizontal pair shows the same field. TyrOHase$^+$ cells (a) are in a group of TyrOHase$^-$ cells stained with Hoechst dye (b). SP$^+$ cells (arrows) (c) stained by HNK1 are surrounded by HNK1$^+$/SP$^-$ cells (d). Two VIP$^+$ cells (arrows) (e) are stained by HNK1 in a group of HNK1$^+$/VIP$^-$ cells (f). Two cells contain TyrOHase (g) and SP (h). (For a, b, c, and d, ×300; for e and f, ×400; for g and h, ×440.)

and HNK1$^-$ cells. In 7 clones, we observed characteristic formations of procartilage that displayed metachromaticism when stained with toluidine blue and were surrounded by HNK1$^+$ and HNK1$^-$ cells. The most frequent clones without neurons were those composed of apparently identical cells that were stained by HNK1 and were similar to those present in clones with neurons (61%) and clones that contained a mixture of HNK1$^+$ and HNK1$^-$ cells (30%).

These experiments thus revealed at least 11 types of colonies. The majority were large clones containing more than one kind of cell. However, the cells were cultured in conditions favorable for the growth of the colonies but in which detection of very small clones of <10 cells is difficult. For this reason, single cells were seeded on 3T3 cells in

Terasaki plate wells (≈1 mm^2), and clonal cultures were fixed and analyzed at 7 days. This preliminary experiment with 70 clones revealed, in addition to the various types of colonies previously observed, some very small clones consisting of only 1–3 neuron-like cells stained with the HNK1 mAb (Fig. 5).

DISCUSSION

Experiments carried out *in vivo* have demonstrated the large range of developmental potentialities of NC cells along the neuraxis and the critical role of environmental factors in their differentiation (1, 3). However, although this pluripotentiality is undoubtedly a property of the NC cell population at all levels of the body, the developmental capacity of individual cells remains a question. This is an important problem since it pertains to the segregation of cell lineages and the mode of action of the environmental factors affecting NC cell differentiation. Does the environment select committed cells able to respond to survival and growth factors or do growth factors exert an instructive effect on a pluripotent cell by driving it through a particular differentiation pathway?

A direct approach to this problem entails the characterization of the progeny derived from single NC cells. Here we describe an analysis of clones obtained *in vitro* by culturing individual NC cells taken when they are undergoing their lateroventral migration at the mes-metencephalic level of 9- to 13-somite quail embryos. For this type of approach to be valid, the culture conditions should ideally provide all the factors required to allow the cells to express the complete spectrum of phenotypes that they have the ability to produce. In this respect, the *in vitro* system described by Green and colleagues (13, 14), in which growth-inhibited mouse 3T3 fibroblasts were used to culture human keratinocytes, has proved to be the most effective for growing cycling NC cells. This was observed with mass cultures of dissociated NC cells, whose proliferation was strikingly higher than in any other culture conditions that we have used (25). In addition to non-neuronal and pigment cells, a number of neuronal cell types differentiated in these cultures, including cells in which TyrOHase colocalized with SP or VIP, whose existence in NC cultures had not been described. Coexpression of VIP and TyrOHase has, however, been documented in cultures of quail dorsal root ganglion cells (22). On the other hand, the expression of SP and TyrOHase in the same cell has not been shown in avian systems, although it has been reported in cultured sympathetic neurons from newborn rat (23).

These particularly favorable culture conditions enabled us to clone NC cells. In the previous attempts to clone NC cells (5, 9–12), colonies were obtained by the limit-dilution method and clonal efficiency, probably difficult to evaluate, was never clearly mentioned. The clones that we obtained were derived from single cells selected individually under microscopic view. It has to be noted that the cloning efficiency was, in the best case, 41% with a mean value of 24%. This very likely reflects the fragility of these young embryonic cells, only a subset of which is able to survive the various manipulations. Consequently, although our results are representative of a large fraction of the population, they may not reflect the developmental capacities of the entire mesencephalic NC. The culture conditions that we have defined are not necessarily favorable for the development of all the progenitors contained in the NC. We have demonstrated (15) that the NC contains post-mitotic neuronal precursors that extend neurites without dividing when cultured in a serum-free fully defined medium. Even low serum concentrations inhibit this process; therefore, such precursors would not be expected to differentiate under the conditions used in the present study. However, on a 3T3 feeder layer and in serum-containing medium, tiny clones composed of only a few neurons could be detected in small wells. They probably originate from precursors other than those arising in serum-free medium. In the present state of our investigations, the number of terminally committed neuronal progenitors present in the mes-metencephalic crest at the stage considered cannot be ascertained.

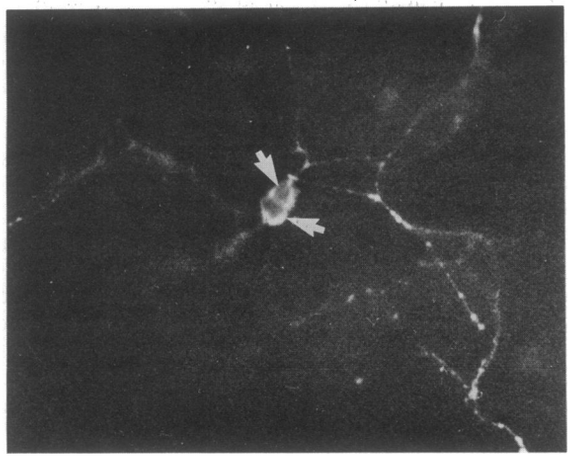

FIG. 5. HNK1 staining of a 7-day clone composed of only two cells with a neuron-like morphology. (×320.)

The most notable result of the present study was identification of pluripotent cells in the NC. Large clones (>20,000 cells), resulting from at least 14 mitotic cycles (which is an underestimate since neuron progenitor cells have stopped dividing early), were commonly found, most of which consisted of several cell types. In particular, neuron-like cells were found in 44% of these clones, 40% of which contained adrenergic ($TyrOHase^+$) cells. Interestingly, the most frequent type of neuronal clone contained adrenergic cells and SP^+ cells. Non-neuronal cells were always found in neuron-containing clones. A common precursor for neuronal and non-neuronal lineages of the peripheral nervous system is thus demonstrated. Furthermore, stem cells whose progeny become melanocytes also give rise not only to adrenergic cells, as reported (5, 10), but also to other neuronal and non-neuronal phenotypes. In contrast to results obtained with truncal NC (5, 9–12), we did not find evidence for a committed pigment cell precursor, suggesting that this particular precursor either does not exist in mes-metencephalic NC at the stage considered or cannot develop in these culture conditions.

We never observed procartilage formation in neuron-containing clones, which could confirm an early segregation of the mesectodermal lineage (2, 24). In contrast, procartilage was found along with $HNK1^+$ cells of non-neuronal morphology. However, procartilage also derives from mesoderm and, since perichondrium can be stained by HNK1 (20), only the simultaneous presence in a clone of an additional well-characterized neurectodermal derivative would unambiguously indicate its NC origin.

In conclusion, the use of growth-inhibited 3T3 cells as a feeder layer and the isolation of single cells by micromanipulation constitute a real advance in our ability to analyze the commitment and developmental potencies of NC cells. This study clearly reveals that the NC is highly heterogeneous with respect to the proliferative ability and developmental potentials of its component cells. The precursors of several types of clones we have observed are probably more or less developmentally restricted members of the same lineage family. This would be consistent with the hypothesis that NC cells become progressively committed during the migration process.

We thank Prof. H. Green and Dr. Y. Barrandon for providing 3T3 cells and precious advice for the cloning procedure; Drs. M. Fauquet and C. Ziller for the gift of the anti-TyrOHase antibody; Dr. J. Smith for critical reading of the manuscript; E. Bourson for typing the manuscript; and Y. Rantier for the illustrations. This work was supported by the Centre National de la Recherche Scientifique, the Fondation pour la Recherche Médicale Française contre le Cancer, a basic Research Grant (1-866) from the March of Dimes Birth Defects Foundation, and the Fonds National Suisse pour la Recherche Scientifique.

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